# How Continuous Immunizations Affect B-Cell Immune Response to Influenza through Cross-Reactive Antibody Expression In Normal-Weight, Overweight, and Obese Individuals.

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## Abstract

**Introduction**: The number of obese individuals has been steadily rising for the last few decades and has reached epidemic proportions. This means that health complications from obesity and being overweight have also increased for all age groups, but specifically for adults who are ages 60 and over. One complication of obesity is the increased risk of mortality from the influenza infection, a deadly illness caused by the influenza virus.

**Goal**: The goal of this study was to determine whether repeat vaccinations increases immunoglobulin antibody response in normal, overweight, and obese adults. It was also to determine whether or not race and age differences have an effect on antibody levels for each weight category.

**Methods**: To accomplish this, serum was collected from subjects both pre and post inoculation with that year's vaccine and an indirect enzyme-linked immunosorbent assay (ELISA) was used to determine antibody levels.

**Results**: Total antibody response showed no significance in any immunoglobulin antibody categories based on weight status alone, but IgG1 and IgG total were significant based on the relationship between time and weight status. African Americans showed higher responses for IgG1 in healthy and obese individuals and showed significantly higher responses in IgG2 overweight individuals compared to Caucasians. Adults ages 30 to 60 showed higher responses in IgG1 for healthy and obese categories compared to adults ages 60 to 90 as well. Obese subjects of all races and ages showed decreased antibody response over time.

**Discussion**: It was determined that continuous immunization could improve antibody responses for the IgG1 and IgG total categories but may not improve other immunoglobulins. We also found that obese people have a diminished antibody response independent of race and that repeat vaccinations help obese people less over time than healthy people and that younger and people have a better immune response at all time periods.

## Introduction

### 1. The Modern Obesity Epidemic

Obesity has reached epidemic proportions recently and levels have been significantly changing for the past decade or more[1]. Adults in nearly every age category have become more obese, especially adults over 60. Categorically, the number of adults over 60 in the obese BMI category has increased 4.4 percent from 2003-2004 to 2011-2012 while adults as a whole (age ≥20) have gained 2.8 percentage points during the same time period[1]. Additionally, Non-Hispanic Blacks have the highest age-adjusted rates of obesity (47.8%) while Hispanics follow with an obesity rate of 42.5%. Non-Hispanic Asians have the lowest with only 10.7% as of 2011-2012[1,2]. Increasingly alarming are the rates of obesity in individuals who are age 19 and below. Although the prevalence of obesity in this age range has remained relatively stable since 2003, it remains high at 17% or 12.7 million people[3]. Similar to adults, Hispanic children and non-Hispanic Blacks make up the highest two percentage groups with 22.4% and 20.2% respectively[3]. While most people hear the term "obese" or "obesity" on a regular basis, it is rarely defined and explained.

Obesity, according to the Centers for Disease Control and Prevention, is defined as having a Body Mass Index (BMI) of 30 or greater. An individual's BMI is a height and weight based analysis that is used to measure a person's body fat content. It does not measure body fat directly, however, and is not used as a diagnostic tool by physicians since it is an approximation. Physicians use other analytical techniques in order to determine difference in visceral fat and abdominal fat, such as skin-fold thickness measurements, diet evaluations, x-ray machines, or hydrodensitometry. BMI is sufficient for Public Health evaluations due to its cost-effectiveness and ease of measurement, but the National Heart, Lung, and Blood Institute recommends additional measurements in order to further improve studies. These two measures include waist circumference, which measures abdominal fat, and risk factors such as high blood pressure, family history, or physical inactivity.

The high prevalence of obesity means that there will be significant health consequences for millions of people. Obesity is a known risk factor for a high incidence of chronic illnesses including coronary heart disease, stroke, type II diabetes, hypertension, cancer, osteoarthritis,

and sleep apnea[6]. The big three diseases that are life-threatening and affect millions of people every day are Heart/Cardiovascular disease, Type II Diabetes, and Cancer. Also, a study conducted by Calle et al in which more than 1 million adults were studied found that not only were obese individuals at risk for many illnesses but were actually at an increased risk of death. Among subjects with the highest BMI, men were 2.58 times more likely to die in the 14 year follow up than men with the lowest BMI while women were 2.00 times as likely[5]. There are also economic consequences to obesity. Williamson et al estimated in 1998 that the total healthcare costs of obesity were 100 billion dollars, meaning that estimates are now likely to have exceeded this level[7]. While this number is only an estimate, it indicates how huge of a cost obesity can be on the healthcare system and society.

## 2. Influenza Virus in Humans

Influenza is a highly infectious disease that is caused by the influenza virus. It is among both the most common forms of human respiratory infections and the most alarming due to its morbidity and, to a lesser extent, mortality. Influenza viruses are a series of negative sense or negative strand RNA viruses that are a part of the Orthomyxovirus family. Of this family, there are three influenza viruses that affect humans: Influenza A, Influenza B, and Influenza C. Of the three types, Influenza C is the least common and has the ability to infect both humans and pigs. It can cause severe illness and local epidemics, but has been shown to have only mild effects in young children with the average fever lasting approximately 2.88 days[8]. Influenza B is a slightly more common virus that almost exclusively affects humans, the seal being the only other species it can infect. Due to the fact that it mutates at a slower rate than Influenza A, it has less antigenic diversity and is less of a threat to organisms. This fact makes epidemics and pandemics nearly non-existent for the virus.

The most common, morbid, and mortal Influenza virus is the A virus. It is widespread and has been shown to affect humans, pigs, birds, and horses. This virus is sub-categorized based on two proteins found on the surface of the viral envelope: hemagglutinin and neuraminidase. Hemagglutinin is a protein that causes RBC coagulation and there are 18 known types. Neuraminidase is an enzyme that cleaves glycosidic bonds of neuraminic acid and there are 11 known types. The particular strains of Influenza A are named based on these proteins. For example, H1N1 contains hemagglutinin type 1 and neuraminidase type 1[9].

There are three main types of influenza virus infections. The first is seasonal influenza that circulates and cause disease on an annual basis. This form of influenza typically infects people of temperate climates during the winter months and the virus itself is contracted on a person-to-person basis[10]. Because these strains are constantly changing, people can become infected by the flu virus multiple times throughout their lives and vaccines must be constantly altered in order to help halt viral propagation. Pandemic influenza occurs when a viral strain emerges which was not previously circulating among humans and to which humans have little to no pre-existing immunity. These strains cause rapid and severe infection which can lead to millions of new cases in a short period of time. Examples include the 2009 H1N1 infection, which has killed upwards of 18,000 people since its appearance, and the "Spanish Flu" of 1918-1919 which killed 20-50 million people. The third type of influenza virus is the zoonotic or variant influenza which routinely circulate in animals and occasionally infect humans. While these zoological strains may share the name of human viruses, they are distinctly different and are not transmitted between humans easily. Thus, this type of virus is not as highly researched.

Influenza is a very common illness that affects thousands of people every year in the United States. According to the CDC, 16,377 laboratory-confirmed influenza-associated hospitalizations have been reported since October 1, 2014 and this number was last updated on April, 3 2015[11]. In past years, the number of positive laboratory tests ranged anywhere from the tens of thousands to 157, 449 during the 2009-2010 H1N1 pandemic year. The hospitalization rate for the 2014-2015 season is 59.9 out of 100,000 and it is highest for older adults over 65 with a rate of 296.2 out of 100,000[11]. It is the highest for that age group since data collection began in the 2005-2006.

Influenza is an acute respiratory disease that infects the respiratory system. Early symptoms include sudden fever, cough, sore throat, headache, and inflammation of the upper respiratory system. More serious symptoms, such as extreme fatigue/weakness, can last for upwards of 10 days[12]. Vomiting and diarrhea can also occur in children, but these symptoms are less common in adults. In addition to common symptoms, people with underlying conditions, such as cardiovascular disease or diabetes, could experience severe complications if infected. Hemorrhaging bronchitis and pneumonia can develop leading to pulmonary edema, hemoptysis, and even death in a matter of days if left untreated[12].

## 3. Immune Cells and Response to Antigens

As previously noted, Influenza is a highly contagious viral infection of the respiratory system. Most experts agree that it is transmitted from person to person through the formation of liquid droplets from coughing, sneezing, or talking in close proximity. The virus is thought to be spreadable up to 6 feet and the droplets formed by the sick are transmitted into the sinuses or lungs of others thereby infecting them[24]. Additionally, influenza can be transmitted from touching infected surfaces or persons and subsequently touching one's own sinus membranes. Once the virus has been contracted, the body's innate immune system is activated in order to eradicate the infection.

The innate immune system is the first line of defense for the body, which includes specific components such as mucus and collectin proteins that act to slow viral dissemination. Additionally, various immune cells, all of which originate in the bone marrow, are produced in order to eliminate the virus itself from the body. Infected cells recognize the Influenza A virus with a special kind receptor called Pattern Recognition Receptors (PRR's). These receptors are capable of recognizing the main marker of the influenza virus, its viral RNA, and are composed of a mix of toll-like receptors (TLR), retinoic acid inducible gene-I's (RIG-I), and NOD-like receptor family pryin domain containing 3 (NLRP3) proteins[13]. Once identified, the virus initiates the release of various pro-inflammatory cytokines and type-1 interferons that have strong antiviral activity and inhibit protein synthesis in virus host cells thereby limiting viral replication. IFN-β is first released, which through a positive feedback mechanism, also stimulates the release of IFN-α. These type-1 interferons also stimulate dendritic cells (DC's), which lie underneath the epithelial lumen of airways, to present antigens to CD4+ and CD8+ T cells and instigate the body's adaptive immune response[13]. Additionally, alveolar macrophages and natural killer cells become activated and phagocytose and lyse infected cells in order to prevent the spread of virus to other healthy cells[13].

The adaptive immune system is a form of long-lasting memory of specific pathogens that allows the body to quickly eliminate future infections from that same pathogen. There are two main types of cells involved in the adaptive immune system: B cells and T cells. B cells work mainly by secreting antibodies into bodily fluids and these antibodies attack any foreign invaders circulating in the bloodstream[14]. Different B-cells are responsible for producing antibodies

specific to the invading antigen. In order to produce the correct antibodies, B cells must rely on antigen-recognition molecules on their cell surfaces. These receptors, called B cell receptors (BCRs), are membrane-bound immunoglobulins that bind antigens and subsequently uptake the antigen molecules by receptor-mediated endocytosis. With the aid of helper T cells and their cytokines, B cells are induced into cells cycles and form plasma cells, which are the forms that secrete the actual antibodies. In this case, the secreted antibodies are of the Immunoglobulin G family. These antibodies then either bind to the pathogen molecules or recruit other phagocytotic cells to the pathogen once other antibodies have bound to it.

T cells work in tandem with B cells but have some distinct differences. Unlike B cells, T cells don't recognize free floating antigens and their surfaces contain T cell receptors (TCRs) that recognize antigen fragments[15]. In most cases, T cells will only recognize antigens if they are bound to the body's major histocompatibility complexes, or MHC's. In order for T cells to differentiate between the body's own cells and foreign cells, these complexes must present the antigens in "recognizable scaffolding". Humans have a specific form of MHC's and they are called human leukocyte antigens, or HLA[15]. As alluded to above, there are multiple types of T cells in the adaptive immune system that have different roles. Helper T cells, aka $CD4^+$ cells, help B cells differentiate into antibody-secreting plasma cells, activate phagocytes, and activate other T cells for continued immune response. Cytotoxic T cells, aka $CD8^+$ or killer T cells, bind directly to antigens and secrete molecules that destroy the antigen.

### 4. Immunizations and Vaccines

According to Vitetta et al, immunologic memory is defined as "the ability to generate a more effective immune response after a second encounter with an antigen"[16]. Upon secondary infection, the immune response is "more rapid, of greater magnitude, is longer –lived, and is characterized by the secretion of antibody of higher average binding affinity"[16]. This means the immune system of previously infected people rapidly eradicates the virus so as to experience hardly any symptoms whatsoever. In order for the body to generate a continuous immunity, special types of B and T lymphocytes are produced called memory B cells and memory T cells. Memory B cells are former antigen-fighting B cells that can rapidly divide into active plasma cells upon secondary entry of the same antigen. Memory T cells operate under a similar concept

in that their surface receptors are incredibly quick to recognize antigens and an army of $CD8^+$ cells are generated to eliminate the virus.

Vaccines are intended to elicit an immune response to a specific antigen once it enters the body. In order to generate that response, inactivated or weakened forms of the antigen are injected into the individual's blood stream so that immune cells can identify and protect against it without triggering the disease[17]. Vaccines do this by triggering immune cells that will generate memory cells based on the antigen. If an inoculated person is infected with the antigen, an intense antibody and cellular response occurs. Antibody release is triggered upon viral infection which leads to the neutralization of the microbes before entering bodily cells and also tags microbes for removal by $CD8^+$ T cells[18]. Thus, vaccines are designed to shield people from contracting dangerous and deadly infectious diseases through the strengthening of the body's natural immune system.

## 5. Subclasses of Immunoglobulin G and Immunoglobulin M

The immunoglobulin G antibodies are a major component of the adaptive immune response in humans and are secreted by B cells in order to neutralize various infections. While there are multiple types of immunoglobulins, immunoglobulin G represents approximately 75% of total serum Ig's[21]. Additionally, they are the only types of immunoglobulins that can cross the placental membrane and are used in scientific research due to their high specificity towards antigens[21].

The immunoglobulin G antibodies are glycoproteins composed of two heavy or gamma ($\gamma$) chains and two light chains that are connected by disulfide bonds. Differences in the subclasses are based on the different amino acid sequences in the heavy chains and the ratio of different light chains, lambda ($\lambda$) or kappa ($\kappa$) chains. While the primary amino acid sequences are approximately 95% the same for all immunoglobulin subclasses, the main region of variation exists at the "hinge" regions of the Y-shaped antibodies[22]. For example, IgG1 has a hinge region of 15 amino acid residues and is highly flexible to allow for antibodies to bind from all directions. However, IgG2 has a hinge region of 12 amino acids and is missing a glycine residue which combines to prevent it from freely rotating[22]. All four IgG subtypes differ at this region which alters their structure and function and is what makes each different from the other.

Immunoglobulin M is an antibody produced by B cells and is the largest antibody produced. It is the first antibody produced during an immune response to antigens and plays roles in agglutination. IgM is also the third most common antibody produced in immune responses and can exist in two forms, a pentamer and a monomer[26]. Like IgG antibodies, IgM antibodies are held together via disulfide bonds. It is predominantly found in lymph fluid and blood and, because of its early action, can indicate infection if at elevated levels[26].

6. **Possible Differences Between Age, Ethnicity, Weight Status**

Multiple studies have shown that there are potentially different levels of immune response to influenza vaccinations in those of different ages. Goodwin et al conducted a weighted analysis of vaccine response based on different vaccine components such as H1, H3, and B influenza antigens[20]. They determined that vaccine response in older adults over the age of 65 was significantly less than their younger counterparts with a much smaller antibody response. They determined that in the ≥65 age group, the influenza vaccine was only 17-53% effective in protecting against H1, H3, and B antigens and that young, healthy adults have a much better immune response that reaches 70-90% efficacy[20].

There have also been studies conducted that detail the differences in immune response based on ethnicity. Ness et al conducted a study on race-specific differences in allelic variation that lead to the up-regulation of inflammatory cytokines[19]. They determined that African-American women were significantly more likely to express these variations, which upregulated the inflammatory cytokines IL1A, IL1B, IL6, IL18, and TNFA[19]. Thus, African-Americans could potentially have genotypes upregulating their inflammatory immune response. Sheridan et al also found that African-Americans had higher immune responses to influenza vaccinations 1 month post vaccination[23]. This finding supports the findings of Ness et al and this study hypothesized that African Americans would have higher antibody responses than Caucasians.

In addition to the potential differences in the former two categories, weight status also may affect the way people build immune responses to antigens. Sheridan et al found that body mass index (BMI) is, at first, positively correlated with higher secretion of IgG antibodies post inoculation with 2009-2010 trivalent influenza vaccine. However, 12 months post vaccination there was a greater decline in influenza antibodies in people of higher BMI compared to normal

or low BMI. Thus, they determined that obesity could diminish a person's ability to mount a sufficient antibody response to influenza.

## Goal and Hypothesis

### 1. Goals

To determine whether repeat vaccinations increases immunoglobulin antibody response in normal, overweight, and obese adults

To determine whether or not race and age differences have an effect on antibody levels for each weight category

### 2. Hypothesis

Repeat vaccinations will enhance IgG and IgM responses in healthy, overweight, and obese individuals.

Caucasians and older adults will benefit from repeat immunizations

## Methods

### 1. Detailed Influenza-Specific ELISA

An enzyme-linked immunosorbent assay (ELISA) was used to identify the level of influenza-specific antibodies in subject serum. Antibody levels, attached by antigen-antibody binding, were analyzed on 96-welled plates. Diluted in coating buffer, the vaccine antigen was bound to the surface of the wells. Block buffer made of milk was added to all plates in order to cover all of the binding sites and subject serum was added. Post incubation and one round of plate washing, secondary goat anti-human IgG (IgG1-IgG4, IgG total) and IgM antibodies conjugated with horse radish peroxidase were added. These antibodies bound to the primary antibodies of the participant's serum. Post washing, enzyme substrate was then added to catalyze a color change inducing reaction. This color change signal was quantified by optical density readings utilizing an ELISA plate reader set at 450 nm. A higher enzyme activity led to a dark, yellow color (higher optical density), and this was directly correlated with antibody concentration.

The ELISA procedure took three days to complete. Prior to the start of the experiment, optimal dilutions for vaccine antigen, serum, and secondary antibody with horseradish peroxidase were determined. Each subclass of IgG (IgG1-IgG4, IgG total) and IgM was measured, and the optimal vaccine antigen dilution for each antibody was chosen to be 1:160.

| | **IgG 1-3** | **IgG 4** | **IgG Total** | **IgM** |
|---|---|---|---|---|
| Vaccine Antigen | 1:160 | 1:160 | 1:160 | 1:160 |
| Sample Serum | 1:800 (in triplicate) | 1:200 (in triplicate) | 1:2000 (in triplicate) | 1:40,000 (in triplicate) |
| Conjugate | 1:1000 | 1:500 | 1:1000 | 1:1000 |

**Table 1.** Standard dilutions used for individual antibodies throughout the experiment

| **Step** | **Serum Sample (µL)** | **Dilution Buffer (µL)** | **Diluted Sample** | **Final Dilution** |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 1 | **10** | 990 | **DS1** | 1:100 |
| 2 | **500 of DS1** | 500 | **DS2** | 1:200 |
| 3 | **250 of DS2** | 750 | **DS3** | 1:800 |
| 4 | **300 of DS3** | 750 | **DS4** | 1:2000 |
| 5 | **50 of DS4** | 950 | **DS5** | 1:40,000 |

**Table 2.** The dilutions of secondary antibodies and serum dilutions used during ELISA runs for IgG1, IgG2, IgG3, IgG4, IgM, and IgG total.

| **Antibody Name** | **Antibody (µL)** | **PBS/BSA (mL)** | **Final Dilution** | **Plate** |
|---|---|---|---|---|
| IgG-1, 2, 3, Total, IgM | **5.5** | **5.5** | 1:1000 | A, B, C, E, F |
| IgG-4 | **11.0** | **5.5** | 1:500 | D |

**Table 3.** The dilutions of goat anti-human IgG conjugated to horseradish peroxidase in dilution buffer

## 2. Specific Details for Antibody Level Analysis Using Enzyme-Linked Immunosorbent Assay

Materials used:

Clear 96 well Falcon plates

Adhesive plate covers

Multi-channel precision pipettes with disposable plastic tips

Plate Reader

Reagents used:

| | |
|---|---|
| Coating Buffer | 0.2 M sodium carbonate/bicarbonate solution in PBS |
| Wash buffer | 330 mL phosphate buffer solution, 3000 mL of distilled $H_2O$, and 1.6 mL of Tween-20 |
| Block Buffer | 2.4 grams of 3% nonfat dry milk in 80 mL of Coating Buffer |
| Dilution Buffer | |
| Detection/Secondary Antibody | Goat anti-human antibody for IgG1, IgG2, IgG3, and IgG4 with horseradish peroxidase conjugate (PBSt) |
| Vaccine Antigen | Diluted in Coating Buffer – 1:160 dilution |
| Horseradish Peroxidase | Attached to detection antibody acting as enzyme conjugate |
| Enzyme Substrate | TMB Substrate in peroxide solution |
| Stop Solution | 2M sulfuric Acid |
| Primary Antibody | Antibodies of study participant serum |

An enzyme-linked immunosorbent assay was used to identify specific IgG and IgM antibodies in the sample serum. Vaccines were first diluted according to Table 1 in coating buffer. Six different 96-well Falcon Plates were used. Once diluted, the plates were allowed to refrigerate overnight at 4°C.

The following day, plates were removed from refrigeration and coated with a milk-based block buffer and allowed to incubate for one hour at 37°C. Post incubation, plates were washed 3x2 times with PBSt using and plate washer. The plates were then coated with milk-based dilution buffer solutions that were made in a 5-step dilution process outlined in Table 2. Plates for IgG1, 2, and 3 were filled with Diluted Solution 3 while the plate for IgG4 received Diluted Solution 2. IgM and IgG total plates received Diluted Solutions 4 and 5 respectively. The plates were incubated at 37°C for 2 hours and washed again 3x2 times with PBSt using a plate washer.

Diluted goat anti-human IgG conjugated to horseradish solution was made according to Table 3 and added to each plate based on IgG subtype. IgG-1, 2, 3, total, and IgM received a 1:1000 diluted solution while IgG-4 received a 1:500 diluted solution. All plates were then washed with PBSt on a plate washer. All plates then immediately received a mixture of TMB solution and peroxide solution. All plates were then incubated at room temperature for 30 minutes under a sheet of aluminum foil. The reaction on the plates was then stopped by adding 100 μL of 2M Sulfuric Acid to each well. Absorbance was then measured with an ELISA plate reader at 450 nm and data was recorded in Microsoft Excel.

3. **Data Analysis**

Using Graphpad Prism 6, data for all antibodies was analyzed and graphs were created. T-tests and ANOVA tests were conducted for all Immunoglobulins (IgG1-IgG4, IgM, IgG total) and graphs were generated based on raw data. Any subjects missing sera or wells, two in this case, were eliminated from the overall study. All subjects' data was placed into Microsoft Excel and separated based on Race, Age, Sex, and Weight Status. Subjects were separated between African-American and Caucasian, years 30-60 and 60-90, male and female, and between healthy, overweight, and obese. Post data separation, percent increase was calculated between Pre- and Post-inoculation for the years 03, 04, and 05. This was calculated by the formula:

$$Percent\ Increase = \frac{Post\ Vaccination\ Value - Pre\ Vaccination\ Value}{Pre\ Vaccination\ Value} x\ 100$$

Mean percent increase was then calculated using Microsoft Excel for all subsets of data. Means were then put into graphs based on data subsets. Two-sample t-tests assuming unequal variances were then conducted to determine significance between data subsets.

## Results

### 1. Study Demographics

Participants in the study were classified into multiple subcategories including sex, race, age, and BMI in order to further compare data. Past studies mentioned have previously shown there is potential for differences in immune response and antibody production between men and women,

African-Americans and Caucasians, and younger and older individuals. Thus, it was worth separating data based on these subcategories in addition to the primary focus that was healthy vs. obese individuals.

| Subject Number | Sex | Race | Age | BMI # | BMI Category |
|---|---|---|---|---|---|
| 03-982 | F | Caucasian | 31 | 18.8 | Healthy |
| 03-970 | F | African-American | 46 | 24.2 | Healthy |
| 03-819 | F | Caucasian | 60 | 21.6 | Healthy |
| 03-955 | F | African-American | 36 | 22.8 | Healthy |
| 03-901 | M | Caucasian | 78 | 24.8 | Healthy |
| 03-588 | F | Caucasian | 69 | 33.3 | Obese |
| 03-971 | F | African-American | 41 | 35 | Obese |
| 03-895 | F | African-American | 49 | 36.2 | Obese |
| 03-788 | M | Caucasian | 69 | 32.5 | Obese |
| 03-856 | M | African-American | 37 | 37.4 | Obese |
| 03-811 | F | African-American | 71 | 25.5 | Overweight |
| 03-993 | F | Caucasian | 61 | 27.4 | Overweight |
| 03-863 | F | Caucasian | 73 | 26.6 | Overweight |
| 03-579 | F | Caucasian | 85 | 26.2 | Overweight |
| 03-707 | F | African-American | 49 | 27.1 | Overweight |
| 03-881 | F | Caucasian | 57 | 26.2 | Overweight |

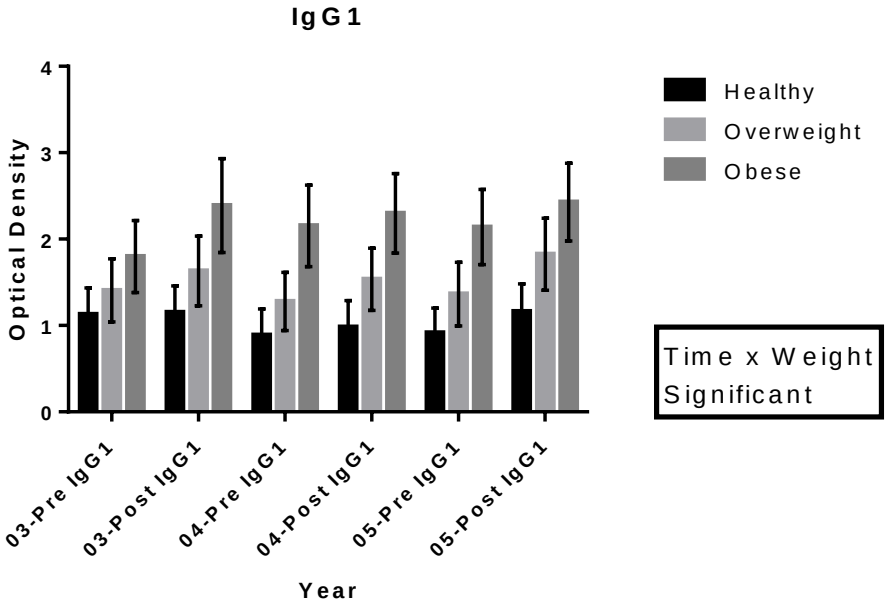
| | | | | | |
|---|---|---|---|---|---|
| 03-927 | F | Caucasian | 55 | 26.4 | Overweight |
| 03-954 | F | African-American | 46 | 28.1 | Overweight |
| 03-631 | M | Caucasian | 64 | 27.4 | Overweight |
| 03-518 | M | African-American | 59 | 27.3 | Overweight |
| 03-820 | M | Caucasian | 62 | 27.5 | Overweight |
| 03-889 | M | Caucasian | 58 | 26 | Overweight |

**Table 4.** The available demographics for all study participants.

2. **Differences in Total Antibody Response**

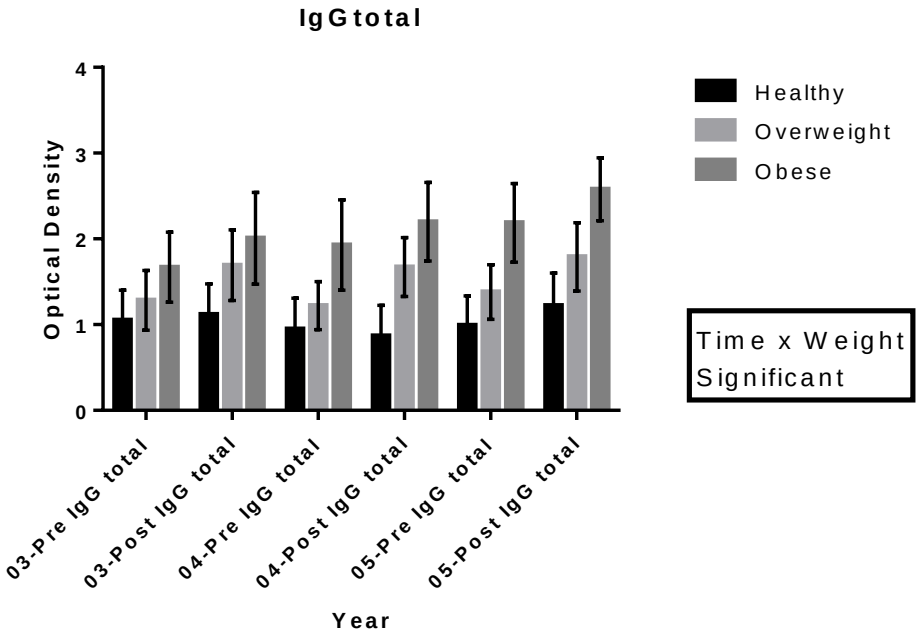
The null hypothesis was that obese individuals will have an inhibited immune response to the H1N1 virus compared to healthy weight people. Both t-tests and analysis of variance tests (ANOVA) were conducted for each individual antibody.

For IgG1, it was determined that there was no relationship based solely on weight status. However, the data were significant for time, subject, and the interaction between weight status and time. For the interaction between time and weight status, a P-value of 0.0168 was calculated with a 0.6770% of total variation. These values were enough to indicate significance as they were under the threshold of 0.05. Both the variables Time and Subjects were also significant and had p-values <0.0001. None of the T-tests conducted for IgG1 came up significant.




Graph 1. IgG1 antibodies for all Healthy, Overweight, and Obese subjects with both pre and post vaccination levels, showing significance between time and weight status

IgG total antibody data was also significant for time, subject, and the interaction between time and weight status. It was also not significant based on weight status alone. For the variable Interaction, the p-value was calculated to be 0.0263, which was enough to be below the significance threshold of 0.05. The percent of total variation was also calculated to be 1.199%. Time and Subject were found to be significant as well with both variables having p-values <0.0001.

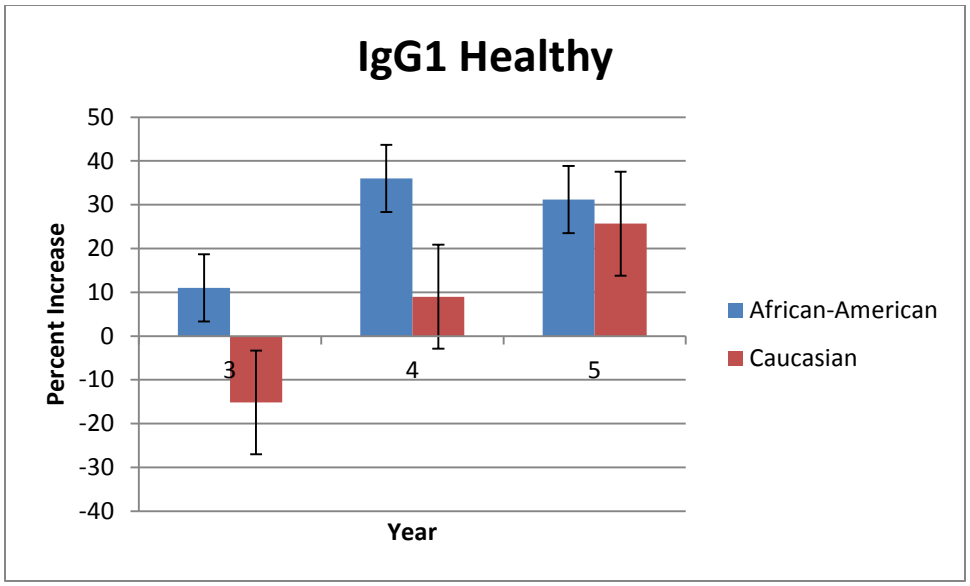



**Graph 2.** IgG total antibodies for all Healthy, Overweight, and Obese subjects with both pre and post vaccination levels, showing significance between time and weight status.

### 3. Percent Increase in Immunoglobulin Response over Time in African-Americans vs. Caucasians

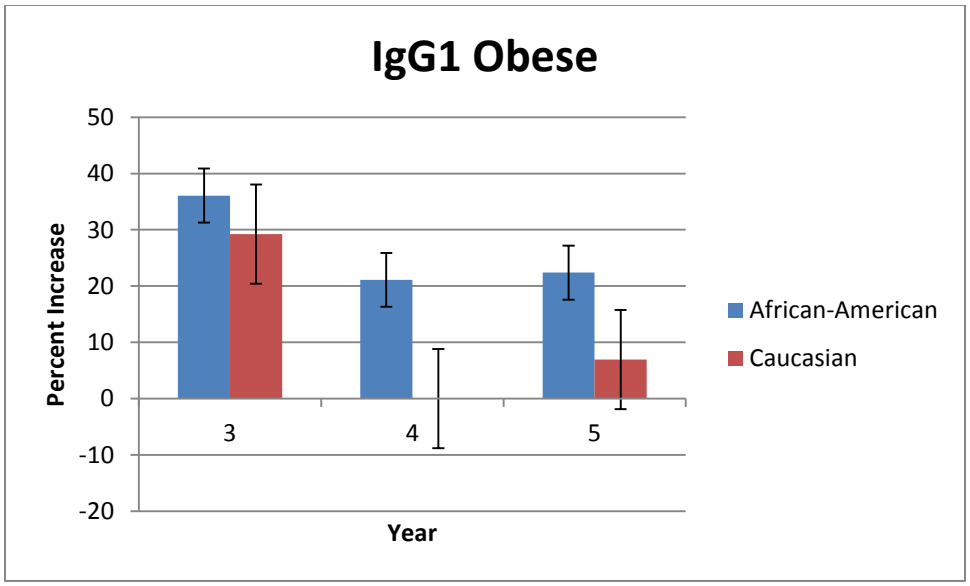
The data was further subcategorized in order to study the percent increase in optical density between pre and post vaccination. The formula discussed in the methods section was used in order to generate these calculations and the mean changes for each subcategory were also found.

For IgG1, the healthy (BMI<25) African-American subjects had a mean change of 26.07% with a variance of 1.76 for all three study years. The sample size for this group was 2. Caucasian subjects had a mean change of 6.49% with a variance of 4.22. The sample size for this group was 3. The p-value of the two groups was found to be 0.26. This was slightly above the threshold for significance of 0.05. The graph for these two groups is shown below in Graph 3.



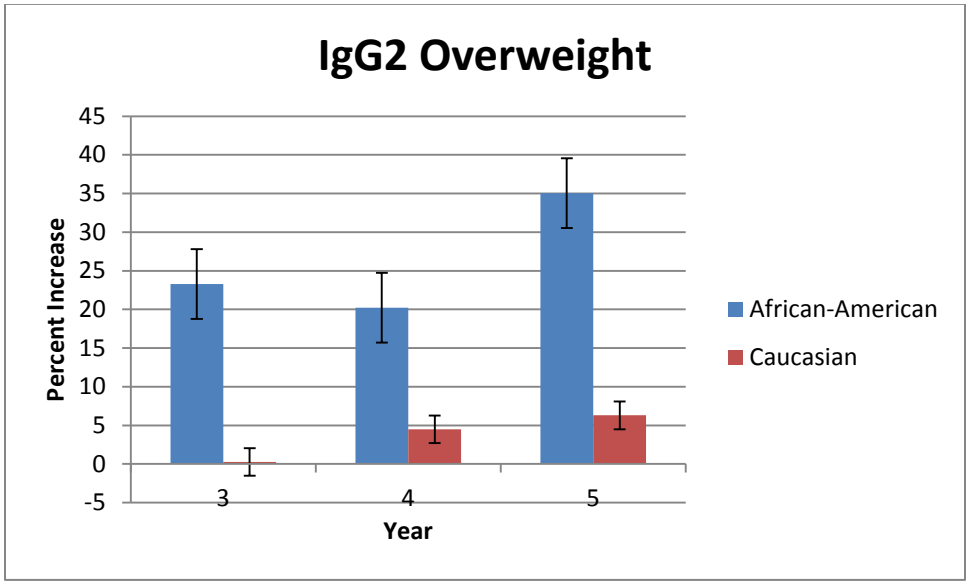

**Graph 3.** IgG1 antibody levels for healthy African-American and Caucasian subjects indicating a 26.07% increase and 6.49% increase respectively.

For obese African-Americans, IgG1 antibody levels in serum increased at an average rate of 26.50% over three years with a variance of 0.689. The sample size of this group was 3. For obese Caucasians, IgG1 serum antibodies increased at an average rate of 11.88% with a variance of 2.40. The sample size for this group was 2. The p-value between the two groups was found to be 0.245. This p-value was slightly above the 0.05 threshold. The graph for these two groups is displayed below in Graph 4.



**Graph 4.** IgG1 antibody levels for obese African-American and Caucasian subjects indicating a 26.5% and 11.88% increase respectively.

For overweight African-Americans, IgG2 antibody levels in the serum increased at an average rate of 26.19% with a variance of .613. The sample size of this group was 4. IgG2 serum levels for overweight Caucasians increased at an average rate of 3.68% with a variance of 0.095. The sample size of this group was 8. The p-value for the two groups was found to be 0.0189. This p-value is below the 0.05 threshold and is therefore significant. The graph for the two groups is shown below in Graph 5.

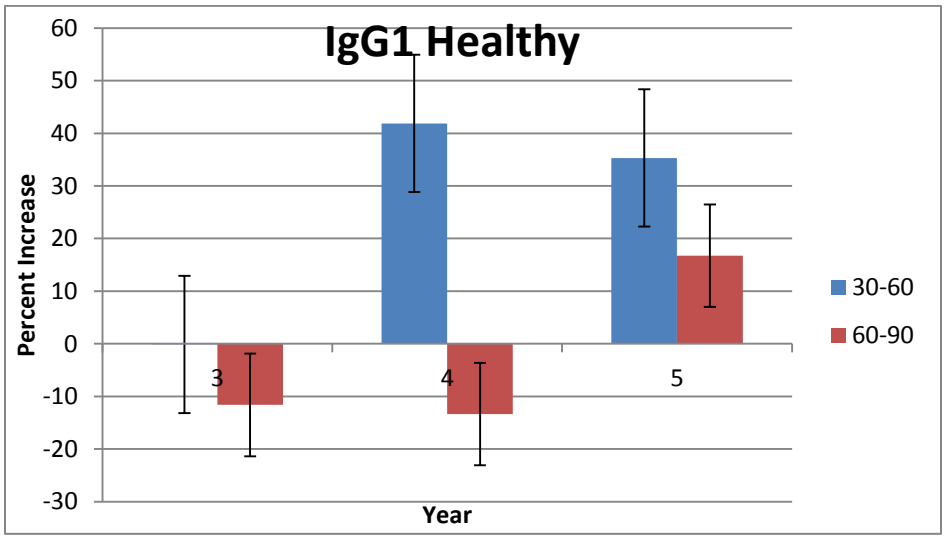



**Graph 5.** IgG2 antibody levels for overweight African-American and Caucasian subjects indicating a 26.19% increase and a 3.68% increase respectively.

### 4. Percent Increase in Immunoglobulin Response over Time in Younger and Older Adults

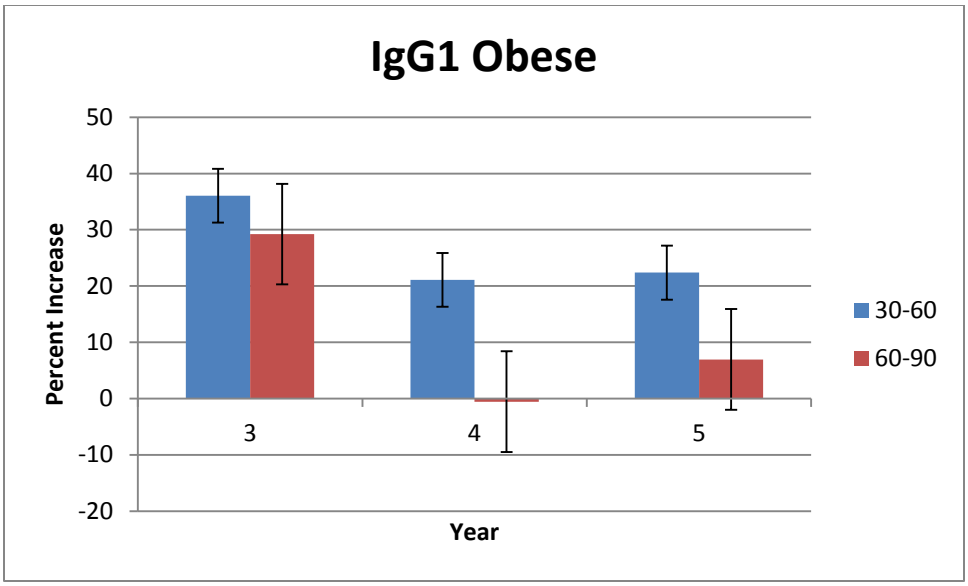
The data was further subcategorized in order to study the percent increase in optical density between pre and post vaccination. The formula discussed in the methods section was used in order to generate these calculations and the mean changes for each subcategory were also found. There were two subcategories created based on age: a 30-60 years group and a 60-90 years age group.

IgG1 serum antibodies increased at an average rate of 25.69% with a variance of 5.10 for healthy individuals ages 30-60. The sample size for the group was 3. For subjects ages 60-90, IgG1 serum antibodies decreased at an average rate of 2.74% with a variance of 2.85. The sample size for this group was 2. The calculated p-value for the two groups was 0.156, which was slightly greater than the 0.05 threshold for significance. The two groups are shown in Graph 6 below.




**Graph 6.** IgG1 antibody levels for healthy subjects ages 30-60 and 60-90, indicating a 25.69% increase and a 2.74% decrease respectively.

IgG1 Serum antibodies for obese individuals ages 30-60 increased at an average rate of 26.50% with a variance of .686. The sample size for this group was 3. IgG1 serum antibodies increased at an average rate of 11.88% with a variance of 2.40. The sample size for this group was 2. The p-value for the two groups was calculated to be 0.245. This is slightly greater than the significance threshold value of 0.05. A graph of the two groups is shown below in Graph 7.




Graph 7. IgG1 antibody levels for obese subjects ages 30-60 and 60-90, indicating a 26.50% increase and a 11.88% increase respectively.

## Discussion

### 1. Conclusions

This study hypothesized that repeat immunizations would enhance the immunoglobulin responses for healthy, overweight, and obese individuals. The total antibody levels for IgG1 and IgG total were found to be significant based on the relationship between time and weight status. This finding is significant because it could mean that continuous immunization could improve antibody responses for the IgG1 and IgG total categories. However, it is also important because the other immunoglobulin categories, IgG2, 3, 4, total, and IgM were not found to be significant. Thus, there is potentially a positive correlation between antibody response and repeat vaccinations, but only for specific antibodies.

It was also hypothesized that Caucasians would benefit from repeat vaccinations so as to be on par with their African-American counterparts. This was found to be only selectively true. While African-Americans had higher percent increases for nearly every category of IgG, Caucasian healthy people had decent increases in antibody concentrations over time. **Graph 3** indicates that while Caucasians actually decreased in antibody response in the first year, they

made large gains in the following two years and came close to the antibody concentrations of African-Americans in the 05 year.

However, this trend is not seen in the overweight or obese categories. The IgG1 antibody response in obese individuals was found to be the opposite of healthy individuals and shows a decreasing level of antibody response after repeat vaccinations. Both African-American subjects and Caucasian subjects show this trend with the Caucasian subjects having much less of a response, though not significant. IgG2 antibody levels in overweight subjects were the only subset that showed a significant difference in percent antibody increase. While there was a slightly positive trend for both races, African-Americans showed a significantly higher response to continuous vaccinations than Caucasians. Thus, while repeat vaccinations may help increase antibody response in healthy individuals of both races, they had little effectiveness increasing antibody responses in overweight and obese individuals with African-Americans having significantly higher responses than Caucasians.

For the age subcategory, there were no tests found to be significant. However, there were trends that indicate potential explanations of the data. The IgG1 data in both healthy and obese subjects were higher for younger adults than older adults and was close to being significant. IgG1 healthy individuals also showed increased immune responses over time in both age categories while IgG1 obese individuals showed the opposite trend, showing less of a percent increase in antibody response over time. This data indicates that repeat vaccinations may help older obese adults less over time than older healthy adults. It also shows that younger adults may have a better immune response at all time periods compared to their older counterparts.

## 2. Improvements for the Future

The best improvement for future studies would be to increase the number of subjects in order to get a better understanding of the population. Because this study only had access to 22 individuals, it was more difficult to analyze and fully understand the data. Having more subjects would most likely solidify the trends that were presented in this paper. It would also help to shrink the width of the age categories in order to better compare age groups. Having additional ethnicities would also give a better understanding of differences in antibody response.

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